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ISOLATION AND SOME PROPERTIES OF 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM *BACILLUS STEAROTHERMOPHILUS**

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SUMMARY

A purification procedure is described for 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate:NADP oxidoreductase (decarboxylating), EC 1.1.1.44) of *Bacillus stearothermophilus* (NCA 1503). A 1200-fold purification was obtained with a 15% yield. The purified enzyme has an approximate mol. wt of 101 000 as estimated by sucrose density gradient centrifugation and consists of subunits of a mol. wt of 51 000. Sulfhydryl group(s) are essential for enzymatic activity as indicated by *p*-chloromercuribenzoate and Ellman's reagent inactivation. Mg^{2+} activate the enzyme at a low concentration (0.01–0.04 M), whereas they inhibit at a higher concentration.

The optimum of the activity was found at about pH 8, with a K_m value at 43 °C of $2.5 \cdot 10^{-5}$ M for NADP and $2.0 \cdot 10^{-5}$ M for 6-phosphogluconic acid. The enzyme is stable at 60 °C, whereas it is inactivated at higher temperatures, with a denaturation half-time of 80 min at 70 °C and 3 min at 80 °C. The enzyme shows a broken Arrhennius plot, with two straight lines meeting at about 50 °C.

INTRODUCTION

Extensive studies on thermophilic enzymes are under way in several laboratories. Knowledge of the properties of a substantial number of enzymes from thermophilic microorganisms will probably be required for a satisfactory understanding of their unusual thermal stability. Besides scientific curiosity, the interest about thermostable proteins is due to the fact that they are to handle during extraction, purification and characterization. Their stability may also led to some practical applications.

In a research program dealing with a general comparison of the properties of enzymes from thermophilic and mesophilic sources, we report here the isolation and some properties of 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate: NADP oxidoreductase (decarboxylating), EC 1.1.1.44) from *Bacillus stearothermo-*

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philus. This enzyme was selected because extensive kinetic and structural information are available for 6-phosphogluconate dehydrogenase isolated from many sources [1–6] and in particular from the mesophilic microorganism *Candida utilis* [7–13].

In addition, since no report has so far appeared on the isolation and characterization of enzymes of the pentose phosphate pathway from *B. stearothermophilus*, this enzyme seemed to be of interest as representative of this metabolic pathway. On the other hand, enzymes of the Embden–Meyerhof pathway, namely fructose-1,6-diphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase and triose phosphate isomerase [14–19] are alrady under extensive investigation.

MATERIALS AND METHODS

Materials

6-Phosphogluconic acid, NADP and NAD were purchased from Boehringer, Mannheim, Germany.

Microgranular DEAE-cellulose (DE-32), phosphocellulose (P-11) and carboxymethyl-cellulose (CM-32) were Reeve and Angel (Clifton, New Jersey, U.S.A.) products, DEAE-Sephadex A-50 from Pharmacia (Uppsala, Sweden), agarose Bio-Gel A-0.5 m and hydroxylapatite Bio-Gel HTP from Bio-Rad (Richmond, California, U.S.A.).

DEAE-cellulose was regenerated with 0.5 M NaOH followed by 0.5 M NaCl and then equilibrated with 0.05 M Tris–HCl, pH 7.2, containing 1 mM EDTA and β-mercaptoethanol (buffer A).

A sample of crystalline 6-phosphogluconate dehydrogenase from *C. utilis* was the generous gift of Dr M. Rippa (Department of Biochemistry, University of Ferrara, Italy).

Growth of B. stearothermophilus

Large cultures of *B. stearothermophilus* (NCA 1503) were grown at 60 °C at the Microbiological Research Establishment (Porton Down, England) following the procedure already reported [20]. The cells were stored at −20 °C.

Enzyme assays

Assays for 6-phosphogluconate dehydrogenase were carried out routinely at 43 °C in semimicro cuvettes of a total vol. of 1.0 ml. The reaction mixture consisted of 6-phosphogluconic acid and NADP ($3 \cdot 10^{-4}$ M each) in 0.1 M Tris–HCl buffer, pH 8.0. The reaction was initiated by the addition of the enzyme solution and followed by the rate of NADPH formation at 340 nm for 1–3 min.

One unit of enzyme activity is the amount of enzyme which is necessary to catalyze the reduction of 1 μmole of NADP/min. Specific activity is expressed as units/mg protein. Protein concentrations were determined spectrophotometrically by the ratio of the absorptions at 280 and 260 nm [21].

When the assays were carried out at temperatures above 43 °C, only temperatures at which the rate of reduction of NADP was linear for at least 2 min were considered. Above 75 °C linearity was not observed.

Malate dehydrogenase (EC 1.1.1.37) [22] and isocitrate dehydrogenase (EC 1.1.1.42) [23] were assayed at 43 °C and fructose-1,6-diphosphate aldolase [24] at 25 °C according to the reported procedures.

Disc gel electrophoresis

Analytical polyacrylamide disc gel electrophoresis was performed at room temperature using equipment similar to that described by Davis [26] and Ornstein [25] at a 10% gel concentration in Tris–glycine buffer, pH 8.5, at 3 mA per tube. Staining was achieved by soaking the gels in a 0.5% solution of amido black in 7% acetic acid and destaining by several washings with the same solvent (minus the dye) or by transversal electrophoresis.

6-Phosphogluconate dehydrogenase activity was detected in the acrylamide gels by tetrazolium reduction. Gels were immersed in 5 ml of Tris–HCl buffer (0.3 M, pH 7.6) containing 1.3 mg NADP, 0.15 mg phenazine methosulfate, 1.5 mg nitro blue tetrazolium and 2.8 mg 6-phosphogluconic acid and then warmed at 60 °C for 1–2 min. After visualization of the bands of enzymatic activity, the brown blue band was fixed in a solution of water–ethanol–acetic acid–glycerine (2:1:1:1, by vol.).

The molecular weight of the 6-phosphogluconate dehydrogenase subunits was estimated by sodium dodecylsulfate polyacrylamide gel electrophoresis following the procedure of Weber and Osborn [27].

The dissociation of the *B. stearothermophilus* enzyme was accomplished by incubating the protein sample in 0.01 M Na_2HPO_4 buffer, pH 7.0, with 1% sodium dodecylsulfate and 1% β-mercaptoethanol for 8 h at 50 °C. The sample was directly applied on top of the sodium dodecylsulfate gel, without prior dialysis.

Density gradient centrifugation

Sucrose density gradient centrifugation was carried out by the method of Martin and Ames [28]. A Spinco Model L ultracentrifuge with a swinging bucket rotor SW 39 was used. The enzyme (0.2 mg in 50 μl) was placed on the top of the 5–20% sucrose gradient in buffer A together with fructose-1,6-diphosphate aldolase (0.1 mg) as internal standard. The gradients were centrifuged for 23 h at 38 000 rev./min at 3 °C. Fractions were then collected and assayed for the enzyme activities.

Inactivation by SH reagents

To a solution of 0.1 mg of 6-phosphogluconate dehydrogenase in 0.3 ml of 0.1 M Tris–HCl buffer, pH 7.2, at 25 °C, *p*-chloromercuribenzoate was added to a final concentration of $5 \cdot 10^{-6}$ M. Samples were removed at the appropriate time intervals and added directly to the standard assay mixture. After 32 min (2% activity remaining), β-mercaptoethanol was added to the reaction mixture to a final concentration of $6 \cdot 10^{-5}$ M. The same experiment was also carried out in the presence of $2 \cdot 10^{-3}$ M 6-phosphogluconic acid.

The inactivation of 6-phosphogluconate dehydrogenase by 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) was carried out in the same conditions as described above but at pH 8.6 with $1 \cdot 10^{-4}$ M reagent. After 23 min (1% activity remaining) β-mercaptoethanol was added to a final concentration of $1 \cdot 10^{-3}$ M.

RESULTS

Purification of the enzyme

All steps were performed at 4 °C.

Step 1: Source of the enzyme. The frozen cells (100 g) were suspended in 300 ml

of 0.05 M Tris–HCl buffer, pH 7.2, 1 mM EDTA and β-mercaptoethanol (buffer A) and subjected to sonic disruption for 30 min at 30 °C by a Branson sonifier (Model B-12). Cell debris was separated by centrifugation at 25 000 $\times$ g for 30 min and the supernatant was removed. The pellet of the cell debris was resuspended in 200 ml of buffer A, stirred for 10 min and again centrifuged as above. The two extracts were combined and solid $(NH_4)_2SO_4$ was added to a 45 % saturation. The precipitate was removed by centrifugation, the supernatant collected and then brought to 75 % saturation with $(NH_4)_2SO_4$. The precipitate from the 45–75 % cut was recovered by centrifugation, dissolved in 50 ml of buffer A, dialyzed overnight against the same buffer and diluted 1:1 with distilled water.

Step 2: DEAE-cellulose. The protein solution from Step 1 was adsorbed on a column (2.5 cm $\times$ 60 cm) of DEAE-cellulose (DE-32) equilibrated with buffer A. Elution was performed at a flow rate of 30 ml/h with 300 ml of the same buffer and then with a linear gradient of 1.2 l of buffer A and 1.2 l of the same buffer containing 0.5 M NaCl. Fractions of 8 ml were collected. The effluent was analyzed for 6-phosphogluconate dehydrogenase activity (Fig. 1A) and at 280 nm for protein concentration.

The elution profile of malate dehydrogenase and of isocitrate dehydrogenase is also reported. It is worthwhile to report that when the proteins were solubilized by French press treatment, the isocitrate dehydrogenase activity was markedly reduced. Both enzymes precipitate in the 45–75 %-$(NH_4)_2SO_4$ saturation cut, but they can be well separated from 6-phosphogluconate dehydrogenase on a DEAE-cellulose chromatography. Thus this procedure can be used for the separation of the three enzymes from the same crude extract without an appreciable loss of activity.

Step 3: DEAE-Sephadex. The pooled fractions from the DEAE-cellulose column having 6-phosphogluconate dehydrogenase activity were dialyzed against 0.50 M Na_2HPO_4, pH 7.0, 1 mM EDTA and 1 mM β-mercaptoethanol. The solution was applied to a column of DEAE-Sephadex (2.5 cm $\times$ 60 cm) equilibrated with the same buffer. The column was eluted with a linear gradient of 1 l of the above phosphate buffer and 1 l of 0.5 M NaCl in the same buffer at a flow rate of 20 ml/h. Fractions of 5 ml were collected (Fig. 1B).

Step 4: Phosphocellulose. The enzyme solution from Step 3 was dialyzed against 0.02 M acetate buffer, pH 6.2, 1 mM EDTA and 1 mM β-mercaptoethanol. The solution was then applied to a phosphocellulose column (1.5 cm $\times$ 10 cm) and equilibrated with the same buffer. The column was washed until no absorbance at 280 nm was observed and then with 100 ml of the same buffer at a 0.2 M sodium acetate concentration. Finally the enzyme was eluted with 0.1 M Na_2HPO_4 buffer, pH 7.2, 1 mM EDTA and 1 mM β-mercaptoethanol. The flow rate was 30 ml/h and fractions of 3 ml were collected (Fig. 1C).

Step 5: Bio-Gel A-0.5 m. The enzyme in the solution from Step 4 was precipitated by dialysis against saturated $(NH_4)_2SO_4$ solution. This precipitation procedure was used since direct addition of solid $(NH_4)_2SO_4$ to a diluted solution of the enzyme at this level of purity was found to partially inactivate the enzyme. The precipitate was recovered by centrifugation and then redissolved in Buffer A and passed through a Bio-Gel A-0.5 m column (1.5 cm $\times$ 100 cm) equilibrated with buffer A. The column was eluted at a flow rate of 10 ml/h and fractions of 1.5 ml were collected. From the active fractions (Fig. 1D) the enzyme was precipitated by dialysis

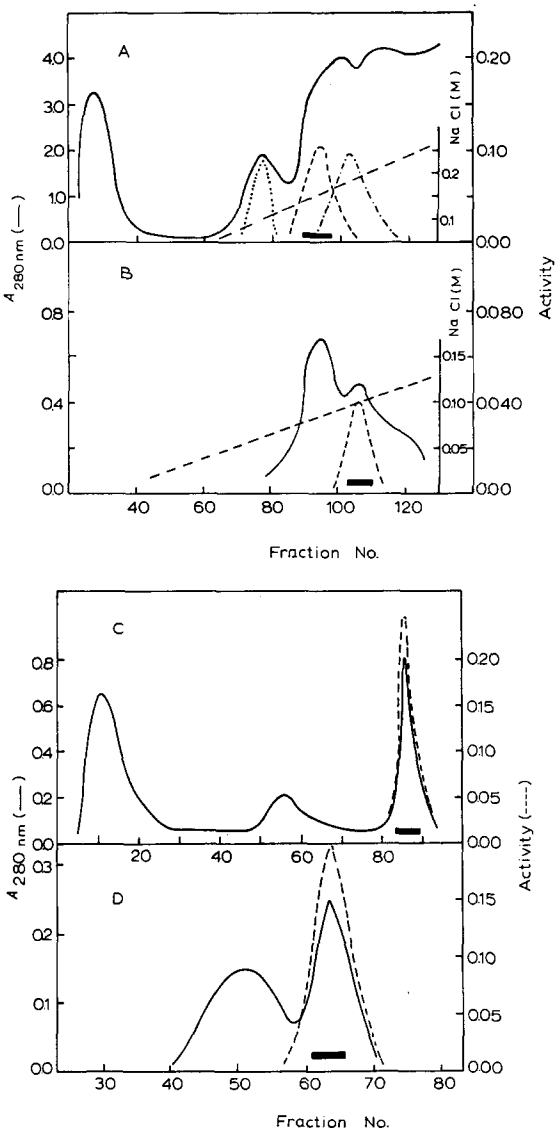

Fig. 1. (A) Elution profile for proteins (———) and 6-phosphogluconate dehydrogenase (– – –) from a DEAE-cellulose column. The elution profile of malate dehydrogenase (· · · · · ·) and isocitrate dehydrogenase (–·–·–) is also reported. (B) DEAE-Sephadex. (C) Phosphocellulose (P-11). (D) Bio-Gel A-0.5 m.

against saturated $(NH_4)_2SO_4$ solution and stored at 4 °C as an $(NH_4)_2SO_4$ suspension.

In Table I a summary of the purification procedure of 6-phosphogluconate dehydrogenase is reported. The enzyme was purified 1200-fold with an overall yield of 15%.

Criteria of purity

The enzyme migrated in analytical disc gels as a single protein band at pH 8.5.

TABLE I

PURIFICATION OF 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM *BACILLUS STEAROTHERMOPHILUS*

Purification step	Volume (ml)	Total units	Specific activity	Purification	Yield (%)
1. Crude extract (from 100 g of cell paste)	500	950	0.07	1	—
2. $(NH_4)_2SO_4$ fractionation	50	870	0.12	1.6	92
3. DEAE-cellulose	65	610	3.10	42	65
4. DEAE-Sephadex	35	560	8.6	120	59
5. Phosphocellulose	15	250	28	380	26
6. Bio-Gel A-0.5 m	7	145	87	1200	15

This single component corresponded to the single band obtained when the gels were stained for 6-phosphogluconate dehydrogenase activity (Fig. 2). A single band was also obtained when the enzyme was dissociated with sodium dodecylsulfate for the determination of the subunit molecular weight.

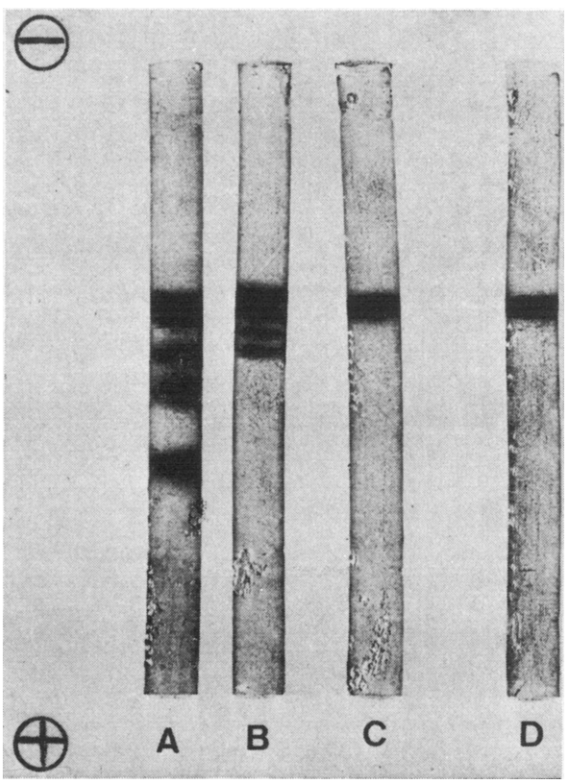

Fig. 2. Acrylamide gel electrophoresis of 6-phosphogluconate dehydrogenase at different stages of purification: (A) After DEAE-Sephadex; (B) After phosphocellulose; (C) After Bio-Gel A-0.5 m, amido black was used for staining; (D) The gel after Bio-Gel A-0.5 m was specifically stained for 6-phosphogluconate dehydrogenase activity.

A spec. act. of 87 I.U. at 43 °C was obtained from several preparations. This value could not be further increased by additional chromatographic runs of the enzyme on hydroxylapatite or CM-cellulose columns, accordingly to similar experiments reported in the isolation of 6-phosphogluconate dehydrogenase from sheep liver [1].

Characterization of the enzyme

To confirm that the enzyme was decarboxylating (EC 1.1.1.44) and not non-decarboxylating (EC 1.1.1.43) the ribulose 5-phosphate end-product of the enzymatic oxidation of 6-phosphogluconic acid was analyzed. Ribulose 5-phosphate was qualitatively identified by paper chromatography as ribulose after enzymatic removal of the phosphate group according to Pontremoli et al. [7]. A quantitative determination was obtained by the pentose-specific chromatic reaction with cysteine–carbazole [29].

Molecular weight

Sucrose density gradient centrifugation was carried out with 6-phosphogluconate dehydrogenase from *B. stearothermophilus* and with the 6-phosphogluconate dehydrogenase Type I from *C. utilis* [9]. Fructose-1,6-diphosphate aldolase was used as an internal reference marker. A typical result obtained with the two enzymes is shown in Fig. 3. Since no difference in sedimentation mobility was observed between the enzymes from the two sources, the same approximate mol. wt can be assumed, i.e. 101 000.

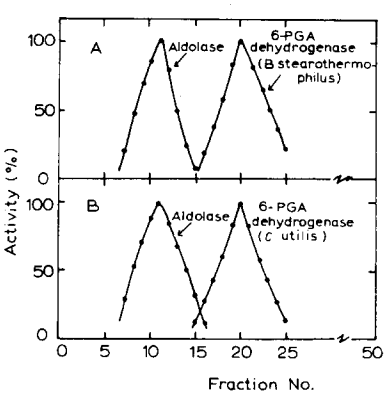

Fig. 3. Sucrose density gradient centrifugation of 6-phosphogluconate (6-PGA) dehydrogenase from *B. stearothermophilus* (A) and of the same enzyme (Type I) from *C. utilis* (B). Fructose-1,6-diphosphate aldolase was used as an internal reference. The ordinates represent the enzyme activities (in arbitrary units) of the fractions collected from the bottom of the 5–20% sucrose gradients.

Fig. 4 shows the determination of the molecular weight of the subunit polypeptide chain after dissociation of the enzyme with sodium dodecylsulfate. One single band was observed after sodium dodecylsulfate treatment with an estimated mol. wt of 51 000.

pH optimum

As shown in Fig. 5 the enzyme exibits a maximum of activity around pH 8 in

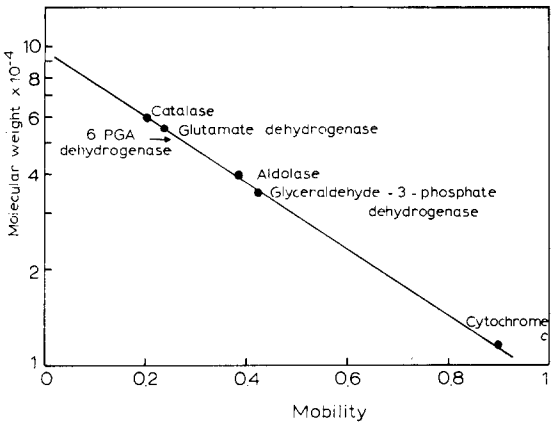

Fig. 4. Determination of the subunit molecular weight of 6-phosphogluconate dehydrogenase by sodium dodecylsulfate disc gel electrophoresis. The mol. wts assumed for the subunits of the marker proteins were: catalase, 60 000; glutamic acid dehydrogenase, 56 000; aldolase, 40 000; glyceraldehyde-3-phosphate dehydrogenase, 36 000 and cytochrome *c*, 11 700.

0.1 M triethanolamine–HCl and Tris–HCl buffers at both 25 and 43 °C. The pH optimum is similar to that found for the *C. utilis* enzyme [11], and about one unit lower of the rat liver enzyme [4].

Kinetics with respect to NADP and 6-phosphogluconic acid

The enzyme follows Michaelis–Menten kinetics with respect to NADP and 6-phosphogluconic acid and shows no substrate inhibition or sigmoidal kinetics in the range of temperatures examined (30–65 °C).

In Table II the apparent K_m values for both substrates at 5 different temperatures are reported; no significant changes in the K_m values have been observed for both NADP and 6-phosphogluconic acid.

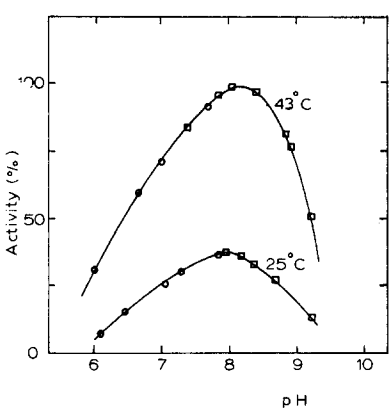

Fig. 5. Enzymatic activity of 6-phosphogluconate dehydrogenase as a function of pH. The buffers used were 0.1 M triethanolamine–HCl buffer (○—○) and 0.1 M Tris–HCl buffer (□—□). The enzymatic activity was assayed in the presence of 0.3 mM of both 6-phosphogluconic acid and NADP at 43 (upper curve) and 25 °C (lower curve).

TABLE II

MICHAELIS CONSTANTS FOR 6-PHOSPHOGLUCONATE DEHYDROGENASE AT VARIOUS TEMPERATURES

(1) Experiments were performed at a constant 0.6 mM NADP concentration and (2) at a constant 0.6 mM 6-phosphogluconic acid concentration, respectively. Other conditions were described in Materials and Methods.

Temperature (°C)	K_m (M)	
	6-Phosphogluconic acid $\cdot 10^5$ (1)	NADP $\cdot 10^5$ (2)
30	1.6	1.7
43	2.0	2.5
45	2.2	2.1
60	1.4	1.1
65	1.4	1.5

The enzyme also exhibits some activity with NAD as substrate and in this case some variation of K_m with the temperature occurs; thus the apparent K_m at 30 °C is $3.5 \cdot 10^{-3}$ M, whereas at 43 °C it is $1 \cdot 10^{-3}$ M. the experiments were carried out with $0.6 \cdot 10^{-3}$ M 6-phosphogluconic acid in both cases. In each case, however, the order of magnitude of the K_m for NAD is 2-fold higher than that observed with NADP and the enzyme may be described as NADP dependent as observed with the already characterized 6-phosphogluconate dehydrogenase from other sources.

Enzyme stability

The enzyme is stable for several days at 4 °C in a solution of buffer A at a low protein concentration (0.1 mg/ml) and for months as an $(NH_4)_2SO_4$ suspension. No loss of activity occurs after lyophilization, whereas some inactivation occurs during ultrafiltration with the Amicon Diaflo membrane (XM-50).

The enzyme partially inactivated on storage can be fully reactivated by incubation at 37 °C in presence of 1 mM EDTA and β-mercaptoethanol.

The stability at high temperatures of the enzyme is largely dependent on the experimental conditions. It was found that samples of the enzyme not purified to the final stage were stable for 2 h at 60 °C in 0.1 M Tris–HCl buffer, pH 7.2.

Analogous stability is shown by the pure enzyme only if warmed in the presence of reducing agents or EDTA. The time course of the inactivation of the enzyme was studied at different temperatures in 0.01 M Na_2HPO_4 buffer, pH 7.1, containing EDTA and dithiothreitol. As can be seen from Fig. 6, the enzyme is stable at 60 °C for at least 2 h, whereas, at higher temperatures inactivation occurs. It was also observed that in the absence of EDTA and reducing agents, casein (1 mg/ml) or the substrate 6-phosphogluconic acid ($3 \cdot 10^{-3}$ M) protect the enzyme from inactivation to some extent.

Inactivation by SH reagents

p-Chloromercuribenzoate in 0.1 M Tris–HCl buffer, pH 7.3, at 30 °C rapidly inactivates the enzyme (Fig. 7A). Partial protection from inactivation is observed when the substrate, 6-phosphogluconic acid, is added to the reaction mixture. The

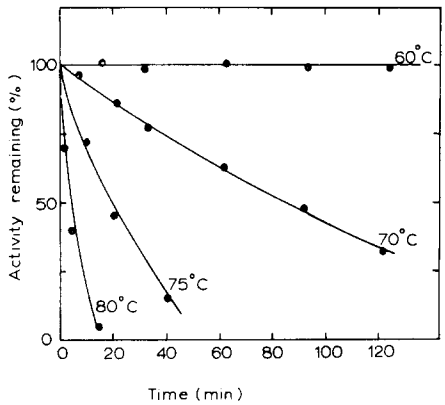

Fig. 6. Heat inactivation of 6-phosphogluconate dehydrogenase from *B. stearothermophilus*. The enzyme (0.1 mg) was dissolved in 1 ml of 0.01 M Na_2HPO_4 buffer, pH 7.1, with 1 mM EDTA and dithiothreitol. The tube was fluxed with N_2, closed and heated at the indicated temperatures. Samples were withdrawn on a time schedule and assayed.

activity is fully and rapidly restored by the addition of β-mercaptoethanol. Analogous reversible inactivation is achieved by Hg^{2+}.

In Fig. 7B is shown the rate of inactivation of the enzyme by 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) and the reactivation by β-mercaptoethanol.

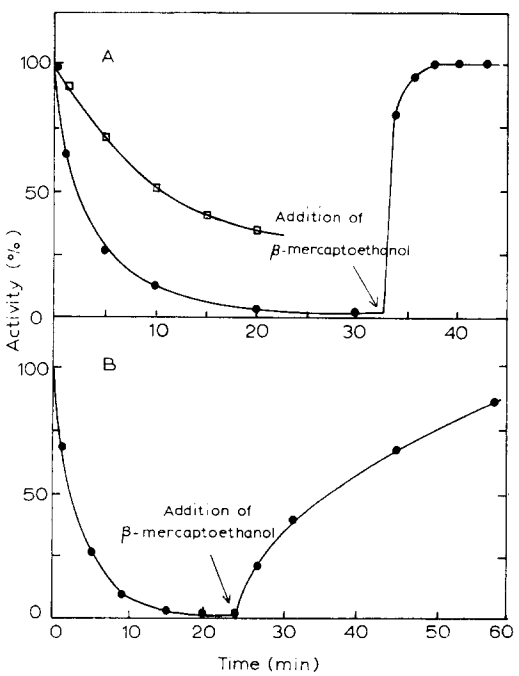

Fig. 7. (A) Time course of the inactivation of 6-phosphogluconate dehydrogenase by *p*-chloromercuribenzoate with (●—●) and without (□—□) the enzyme substrate 6-phosphogluconic acid ($2 \cdot 10^{-3}$ M) and reactivation with β-mercaptoethanol. (B) Inactivation of 6-phosphogluconate dehydrogenase by 5,5′-dithio-bis-(2-nitrobenzoic acid) (Ellman's reagent) and reactivation by β-mercaptoethanol.

Effect of salts

As illustrated in Fig. 8, $MgCl_2$ has an activating effect at low concentrations, whereas at higher concentrations the salt behaves as an inhibitor. The effect is related to the Mg^{2+} since neither KCl, NaCl or sodium acetate show any effect on the enzymatic activity of 6-phosphogluconate dehydrogenase over the range of concentrations examined (0.06–0.2 M).

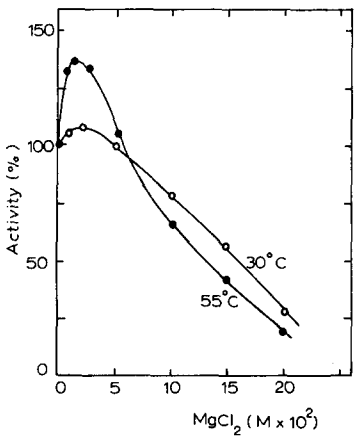

Fig. 8. Effect of $MgCl_2$ concentration on the activity of 6-phosphogluconate dehydrogenase at 30 (○—○) and 55 °C (●—●). The buffer used in the assay mixture was 0.1 M Tris–HCl buffer, pH 8.0, and the concentration of both NADP and 6-phosphogluconic acid was $0.6 \cdot 10^{-3}$ M.

The effect of salts is different from that demonstrated for the *C. utilis* enzyme, where enzyme activity was not only related to Mg^{2+}, but also to the nature of the anion [7]. In fact, NaCl and KCl at high concentrations activate the enzyme.

The activating effect of Mg^{2+} on 6-phosphogluconate dehydrogenase activity is about four times greater at 55 than at 30 °C and, similarly, the inhibition is less pronounced at 30 than at 55 °C.

Effect of temperature on the reaction rate

Fig. 9A shows the effect of temperature on the rate of the enzymatic reaction and Fig. 9B the Arrhenius plot of the same data. The graph has a discontinuity of slope and approximates to two straight lines meeting at about 50 °C. An apparent activation energy of 8490 cal/mole above 50 °C and 11 130 cal/mole below 50 °C was calculated. It was observed that the enzyme purified from the bacterial cells disrupted by French press treatment shows a different arrhenius plot with two straight lines meeting at about 43 °C, with an activation energy of 15 500 and 11 140 cal/mole above and below 43 °C respectively. In addition the linearity of the plot is observed only up to about 58 °C. Differences in behaviour and properties of enzymes related to different methods of solubilisation and purification have been already observed, *e.g.* in the case of bovine liver glutamate dehydrogenase [32].

It must be pointed out that this biphasic relationship is not due to a change in the apparent Michaelis constant for either NADP or 6-phosphogluconic acid since they are nearly identical in the range of 30–65 °C, as reported in Table II. On the other

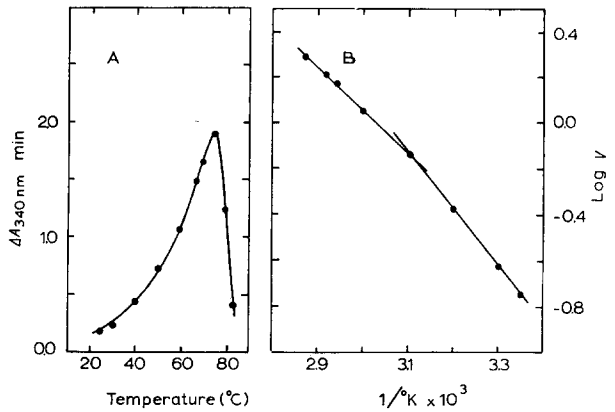

Fig. 9. (A) The effect of temperature on the enzymatic assay of 6-phosphogluconate (6-PGA) dehydrogenase. The assays were performed in 0.1 M Tris–HCl buffer, pH 8.0, with both 6-phosphogluconic acid and NADP at a concentration of $0.6 \cdot 10^{-3}$ M. The standard assay mixture (1.0 ml) was warmed at the indicated temperature and then the reaction started by the addition of 5 μl of an enzyme solution of 87 I.U. spec. act. (B) Arrhenius plot of the data of Fig. 9A. Values above 75 °C were not considered.

hand, since the concentrations used for both NADP and 6-phosphogluconic acid were about 30–40 times higher than the K_m value, it is safe to assume that the data reported in Fig. 9 represent maximal velocities at the respective temperatures.

DISCUSSION

The procedure described in this paper for the purification of 6-phosphogluconate dehydrogenase (decarboxylating) from the obligate thermophile *B. stearothermophilis* results in an electrophoretically homogeneous enzyme with a 1200-fold purification and an overall yield of 15%.

In general the catalytic properties of the thermophilic 6-phosphogluconate dehydrogenase are similar to those reported for the same enzyme isolated from other sources.

The enzyme has a specific activity and K_m values for both NADP and 6-phosphogluconic acid comparable to the figures reported for the *Neurospora* [6] and *C. utilis* (Type I) enzymes [9].

The *B. stearothermophilus* enzyme is inactivated by SH reagents in a similar way to the yeast enzyme [7]. The enzyme is completely inhibited by *p*-chloromercuribenzoate and 5,5′-dithiobis-(2-nitrobenzoic acid) and reactivation is obtained by β-mercaptoethanol treatment; the substrate 6-phosphogluconic acid shows a protective effect on the inactivation. These results indicate the presence of sulfhydryl group(s) at or near the active site of the enzyme, as already observed for the *C. utilis* and *Neurospora* enzyme.

The approximate mol. wt of the native thermophilic 6-phosphogluconate dehydrogenase, 101 000, and of its subunit polypeptide chain, 51 000, has been found similar to the enzyme isolated from the *C. utilis* (Type I enzyme) [9], and from human erythrocytes [5].

On the other hand, 6-phosphogluconate dehydrogenase from *B. stearothermophilus*, like other enzymes from this organism, exhibits higher temperature optima and thermal stabilities than the corresponding enzyme from mesophilic organisms. In fact, the enzyme is stable for at least 2 h at the growth temperature of the microorganism, i.e. 60 °C, whereas for example, the *C. utilis* enzyme is rapidly inactivated. At higher temperatures, the enzyme becomes less stable, the half-time of thermal inactivation being about 80 min at 70 °C, 20 min at 75 °C and 3 min at 80 °C.

Mg^{2+} have an activating effect on the thermophilic enzyme at low concentrations and an inhibitory effect at high concentrations. The extent of both the activation and the inhibition are enhanced by changing the temperature from 30 to 55 °C. This may indicate that the molecular structure of the enzyme at 55 °C is different from that at 30 °C. It is of interest to note that the *B. stearothermophilus* enzyme exhibits a broken Arrhenius plot, with two straight lines meeting at 50 °C. Some examples of similar temperature effects on activators or inhibitors are documented in the literature in the case of other enzymes, including thermostable ones [18, 30, 31].

It will be of interest to relate these results on the catalytic properties of 6-phosphogluconate dehydrogenase of *B. stearothermophilus* with its conformational characteristics. Studies on these lines are under way.

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